Journal of the American College of Cardiology
© 2006 by the American College of Cardiology Foundation
Published by Elsevier Inc.

Vol. 48, No. 6, 2006
ISSN 0735-1097/06/$32.00
doi:10.1016/j.jacc.2006.05.046

The $T^{-786}C$ Endothelial Nitric Oxide Synthase Genotype Predicts Cardiovascular Mortality in High-Risk Patients

Gian Paolo Rossi, MD,* Giuseppe Maiolino, MD,* Mario Zanchetta, MD,† Daniele Sticchi, PHD,* Luigi Pedon, MD,† Maurizio Cesari, MD,* Domenico Montemurro, MD,* Renzo De Toni, BSD,* Silvia Zavattiero, PHD,* Achille C. Pessina, MD, PHD*

Padua, Italy

OBJECTIVES This study sought to investigate the impact of a common $T^{-786}C$ single-nucleotide polymorphism (SNP) in the promoter of the endothelial nitric oxide synthase (eNOS, NOS3) gene on cardiovascular (CV) death in a prospective cohort study.

BACKGROUND The $T^{-786}C$ SNP eNOS gene implies a blunted endothelium-dependent vasodilation in hypertensive patients and was associated with multivessel coronary artery disease in cross-sectional studies, but it remained unsettled whether it carried prognostic information.

METHODS In consecutive white patients of the GENICA (Genetic and Environmental Factors in Coronary Atherosclerosis) study, who underwent coronary angiography between 1999 and 2001, we determined the incidence of CV death at follow-up. The eNOS $T^{-786}C$ and the exon 7 $G^{894}T$ SNPs were determined by melting curve analysis of amplicons from allele-specific fluorescence resonance energy transfer probes. Plasma levels of nitrate/nitrite, nitrotyrosine, and myeloperoxidase were also measured. The Kaplan-Meier and Cox regression analyses were used to assess the impact of SNPs on event-free survival.

RESULTS Complete follow-up data were obtained in 1,086 (98%) patients. After a median follow-up of 1,296 days (range 4 to 2,057 days), we observed 85 (8.2%) CV deaths. There was a significant impact of the $T^{-786}C$ eNOS genotype on CV death-free ($p = 0.0102$) survival, but no differences in CV death rates across $G^{894}T$ genotypes. The TT individuals, who showed a lower survival, exhibited higher plasma myeloperoxidase ($p < 0.0001$) and lower levels of nitrotyrosine ($p < 0.0001$) than CC patients.

CONCLUSIONS The $T^{-786}C$ SNP in the promoter of eNOS bears independent prognostic information and is associated with changes in markers of oxidant stress in high-risk white patients referred for coronary angiography. (J Am Coll Cardiol 2006;48:1166–74) © 2006 by the American College of Cardiology Foundation

The susceptibility to coronary artery disease (CAD), the major cause of cardiovascular (CV) mortality in developed countries, is determined by genetic factors (1); although some accomplishments have been made (2–4), the complex fabric of this common polygenic disorder has been difficult to unravel (5).

See page 1175

Dysfunction of the vascular endothelium, defined as an impaired nitric oxide (NO) bioactivity, which is at least in part genetically determined and can predict CV events (6–9), plays a substantial role in the triggering of atherogenesis as well as in the erosion, or rupture, of atherosclerotic plaques that underlie CV events. The gene of a major NO-forming enzyme in the vasculature, the endothelial nitric oxide synthase (eNOS), entails several single-nucleotide polymorphisms (SNPs), some of which have functional relevance (10). The GAG to GAT substitution in exon 7 ($G^{894}T$) determines the conservative replacement of glutamate with aspartate (Glu298Asp), which might cause a tight turn of the alpha-helix and therefore an increased susceptibility to degradation (11). However, it remains controversial whether this impairs the function of eNOS (12). According to cross-sectional studies, the $T^{-786}C$ SNP located in the eNOS gene promoter has a significant association with a blunted NO bioactivity in vivo in hypertensive patients (13), and with multivessel CAD in Caucasian (14,15) and Japanese patients (16). However, although an impaired NO bioactivity has been consistently associated with accelerated atherosclerosis (17,18) and with CV and cerebrovascular events (6,7), cross-sectional studies can be hampered by several biases and therefore should be taken with caution. Moreover, it remains altogether unknown whether these eNOS SNPs had an impact on CV death- and event-free survival (19).

Thus, within the prospective branch of the GENICA (Genetic and Environmental Factors in Coronary Atherosclerosis) study, we sought to test the hypothesis that the $G^{894}T$ and $T^{-786}C$ eNOS SNPs independently predict CV deaths.

METHODS

Study participants. The study protocol and criteria for enrolment of the patients in the GENICA study were previously detailed and, therefore, will be only briefly recalled

From the *DMCS-Internal Medicine 4 and the †Servizio di Emodinamica and Divisione di Cardiologia Ospedale di Cittadella, University of Padua, Padua, Italy. This study was supported by research grants from Regione Veneto (863/01/98), Unindustria of Treviso, and F.O.R.I.C.A. to Dr. Rossi.

Manuscript received December 23, 2005; revised manuscript received April 13, 2006, accepted May 2, 2006.

Abbreviations and Acronyms

CV = cardiovascular
CAD = coronary artery disease
eNOS = endothelial nitric oxide synthase
HDL = high-density lipoprotein
LDL = low-density lipoprotein
LVEF = left ventricular ejection fraction
MI = myocardial infarction
MMP = matrix metalloproteinase
NO = nitric oxide
RNS = reactive nitrogen species
ROS = reactive oxygen species
SNP = single-nucleotide polymorphism

(14,20). Consecutive Caucasian patients referred for coronary angiography for investigation of chest pain and/or suspected CAD between 1999 and 2001 were enrolled upon signature of a consent form to participate in this study. The study protocol was approved by the medical ethics committee. Refusal to participate was the only exclusion criterion. Information on medical history, smoking habits, presence/absence of arterial hypertension, diabetes mellitus, hypercholesterolemia, hypertriglyceridemia, and current medications was gathered with a staff-administered questionnaire (14). Criteria for defining body mass index, smoking status, diabetes mellitus, impaired glucose tolerance, hypercholesterolemia, and hypertriglyceridemia have already been reported (20,21). Blood pressure was measured by mercury sphygmomanometer using Korotkoff phase V for diastolic pressure, according to the World Health Organization guidelines; arterial hypertension was defined according to the European Society of Cardiology/European Society of Hypertension guidelines criteria, or use of anti-hypertensive agent(s) (22).

Coronary angiography. Angiography and measurement of left ventricular ejection fraction (LVEF) and the grading of the burden of CAD was carried out, as recently described (23). The severity of CAD was graded independently on an ordinal scale on which 0% corresponded to no stenosis and 100% to vessel occlusion by two observers (M.Z. and L.P.) who were blinded to the patient's genotype. The percentage of stenosis was derived by the mean of the visual estimates if the between-estimate difference was <20%; greater inter-observer disagreement in the grading of stenosis was resolved by consensus. The burden of CAD present was summarized using the Duke Prognostic Index (24), modified according to Mark et al. (25) to account for the impact of left main trunk stenosis. This index, which accurately predicted 5-year mortality of medically treated patients, takes into account major epicardial coronary arteries with ≥50% diameter stenoses and also the location within the vessel, based on the premises that proximal coronary stenoses have a stronger impact on prognosis than distal coronary stenoses (26). This score goes from 0 (all major coronary arteries with lesions <50% diameter stenosis) to 100 (≥95% left main stenosis), and therefore provides an estimate of atherosclerotic burden, although it considers only major epicardial coronary arteries with ≥50% diameter stenosis. For the purpose of comparison of haplotype frequencies, patients were classified as non-CAD and CAD if they had a CAD score of 0 or >0, respectively.

Laboratory measurements. Patients were studied between 8:30 AM and 12:00 PM. Blood samples were taken immediately before coronary angiography, put on ice, and centrifuged at 3,000 × *g* (at 4°C for 10 min). Total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glycemia, sodium, potassium, and creatinine levels were measured as described (23). All measurements as well as the measurement of nitrate/nitrite, nitrotyrosine, and myeloperoxidase (see later text) were performed blinded to genotype data.

Extraction of deoxyribonucleic acid and eNOS genotyping. The DNA was extracted from the blood stored at −20°C using commercially available kits. Genotyping at the eNOS polymorphisms was performed with LightCycler (Roche, Milan, Italy) using melting curve analysis from an allele-specific fluorescence resonance energy transfer probe. This method was preferred to the restriction fragment length polymorphism analysis (which showed inconsistent cleavage) because it was found to be 100% accurate when compared with sequencing. Primers and fluorescence resonance energy transfer sequences have already been reported in detail (14).

Nitrate/nitrite assay. Samples of plasma-ethylene diaminetetraacetic acid disodium salt were filtered through a 30-KDa molecular weight cut-off filter using a commercially available centrifuge ultrafiltration device (Millipore, Milan, Italy) to reduce background absorbance caused by the presence of hemoglobin. Assay was performed with a commercial kit (Nitrate/Nitrite; Cayman, Ann Born, Michigan) in 96-well plates using 80 μl of the filtrate. The conversion of nitrate to nitrite, which requires 3 h at room temperature for completion, was achieved by adding 10 μl of the enzyme cofactor and 10 μl of nitrate reductase to the plasma. After incubation, 50 μl of Griess reagent R1 and immediately after, 50 μl of Griess reagent R2 were added to each well and the color was allowed to develop for 10 min at room temperature. The color that developed was measured by reading the absorbance at 450 nm. The absorbance value of the blank wells was averaged and subtracted from the absorbance value of all of the other wells. The nitrate standard curve has been used for determination of nitrite alone. The detection limit is about 2.0 μmol/l.

Enzyme immunoassay for nitrotyrosine. Nitrotyrosine was quantified by a "sandwich" enzyme-linked immunosorbent assay Nitrotyrosine-EIA (OxisResearch, Portland, Oregon). For the assay, aliquots of the plasma samples were diluted 10× with the working dilution buffer provided with the kit and assayed in duplicate. Assay was performed in 96-well plates coated with a solid-phase monoclonal antibody (nitrate keyhole limpet hemocyanin raised in mouse), and the nitrotyrosine cap-

tured was detected with biotin-labeled goat polyclonal anti-nitrotyrosine for 2 h at room temperature, followed by washing the plate with phosphate-buffered saline. Sequential incubations were then performed with biotinylated goat immunoglobulin G and a streptavidin peroxidase conjugate to form avidin-biotin horseradish peroxidase complex. After further washing, color development was initiated by the addition of substrate tetramethylbenzidine and was allowed to develop in the dark for up to 30 min at room temperature and terminated by the addition of 2 mol/l citric acid solution. The yellow product was measured at 450 nm. A standard curve was constructed by incubating in the wells serial dilutions of 4.5 μmol/l nitrotyrosine standard in duplicate. The nitrotyrosine concentrations of the samples were estimated from the standard curve. The intraassay and interassay coefficients of variation were 2.3% and 11.2%, respectively, and the detection limit was 2 nmol/l.

Diacron reactive oxygen metabolites test septic colorimetric assay. Samples of serum were filtered as described above to reduce background absorbance caused by the presence of hemoglobin. Azo-cloro-compounds were measured with the Diacron reactive oxygen metabolites test (D-ROMs Septic Test, Diacrons, Grosseto, Italy). In the presence of peroxides (azo-cloro-compounds), this test is based on the ability to catalyze the formation of free radicals, which are trapped by an alchilamine, resulting in a colored radical that is detectable at 505 nm. Ten microliters of serum were mixed with 198 μl of a pH 4.8 buffer and 2 μl of an alchilamine reagent, using the end point method in the plate reader. The samples were mixed and incubated (75 min; 37°C) and read for optical density. After 10 min, the sample was read again. The average difference in absorbance A is multiplied by the dilution factor and calculated by a standard curve built using serum with defined values. The obtained values were expressed as arbitrary units or CARR U, where 1 CARR U corresponds to 0.08 mg/100 ml H_2O_2.

Follow-up data. Information on the long-term outcome of the patients enrolled in the GENICA study was gathered blinded to the patient's genotype with a predefined form, through review of medical charts for the patients regularly seen at referring hospitals, and through telephone interviews of family doctors and/or patients and first-degree relatives for those not attending regular follow-up visits. The alive/death status was ascertained through on-site investigation for all patients lost to follow-up. The predetermined primary end point was CV death, defined according to the Syst-Eur Trial (27) as sudden or as caused by congestive heart failure, acute coronary syndromes, or stroke, and validated by the adjudication committee (G.P.R. and G.M.) blinded to patients' genotypes and biochemical data. Because the exact date of occurrence of each event was precisely known, survival data are presented in days on the time scale. Information on CV events, including myocardial infarction (MI), unstable angina, stroke, vascular surgery, and coronary revascularization, was also gathered.

Statistical analysis. One-way analysis of variance followed by the Bonferroni post-hoc test was used for comparison across genotypes. The nonparametric Mann-Whitney *U* test was used to compare plasma levels of nitrate/nitrite, nitrotyrosine, and plasma levels of myeloperoxidase between CC and TT patients. Chi-square analysis was used to compare the frequencies of categorical coronary risk factors, medical therapy at baseline, and the SNP across eNOS genotypes and to verify agreement of the genotype frequency with the Hardy-Weinberg equilibrium. The CV death rates were estimated by Kaplan-Meier analysis with the log-rank test. Cox stepwise (backward, Wald) and sequential regression analysis (28) were also used to determine the relationships between CV death at follow-up and several independent variables ($T^{-786}C$ and $G^{894}T$ eNOS SNPs, LVEF, gender, age, arterial hypertension, low-density lipoprotein [LDL] cholesterol, smoking status, serum creatinine, diabetes mellitus, body mass index, Duke Prognostic Index, and treatment).

The modified Duke Prognostic Index was included in the analysis either as a continuous or as a categorical (dichotomized or divided into quartiles) variable. The impact of the $T^{-786}C$ SNP was examined according to a dominant model, e.g., CC + CT versus TT, because the ^{-786}C allele was found to imply a blunted NO bioactivity in both the heterozygous and the homozygous cases (13). Statistical significance was defined as $p < 0.05$. All analysis were performed using SPSS version 13 for Windows (SPSS Italy Inc., Bologna, Italy).

Haplotype analysis. Linkage disequilibrium and haplotype analyses were performed by use of the Thesias program (29) based on the SEM algorithm (30). The Thesias program allows estimation of both haplotype frequencies and covariable-adjusted haplotype effects by comparison with a reference haplotype taken as the most frequent haplotype in the current analyses. A global test of association between haplotypes and any studied phenotype was performed by means of a chi-square test with m-1 degree of freedom in the case of m haplotypes. All analyses were adjusted for gender; hypotheses were tested by means of likelihood ratio criterion. A p value <0.05 was considered significant.

RESULTS

Clinical characteristics and eNOS genotype distribution. Of the patients originally recruited in the GENICA study, 1,104 had informative eNOS genotype data; of them 1,086 (98%) had complete coronary angiography and follow-up information. The coronary angiography findings in the GENICA study high-risk cohort at the baseline evaluation are summarized in Table 1 in the patients divided by $T^{-786}C$ genotype. Overall, in this population of consecutive patients referred for coronary angiography, about 25% had a CAD score of 0, and the rest had a score >0. The demographic and clinical characteristics of these patients

Table 1. Distribution of the Patients in the Categories of CAD Atherosclerotic Burden Estimated With a Modified Duke Index Prognostic Score

Extent of CAD	Prognostic Weight (0–100)	Patients Stratified by $T^{-786}C$ Genotype	
		TT (n = 436, 39.5%)	TC and CC (n = 668, 60.5%)
No CAD ≥50%	0	20.9	27.1
1-vessel disease 50% to 74%	19	6.5	6.4
>1-vessel disease, 50% to 74% or 1-vessel disease (75%)	23	16.4	16.8
1-vessel disease (≥95%)	32	9.3	9.1
2-vessel disease	37	15.5	14.0
2-vessel disease (both ≥95%)	42	1.6	2.9
1-vessel disease, ≥95% proximal LAD or 2-vessel disease, ≥95% LAD	48	10.0	7.0
2-vessel disease, ≥95% proximal LAD or 3-vessel disease	56	3.9	4.2
3-vessel disease, ≥95% in at least one	63	5.3	4.2
3-vessel disease, 75% proximal LAD	67	3.7	3.8
3-vessel disease, ≥95% proximal LAD	74	2.8	1.7
Left main (75%)	82	3.9	2.3
Left main (≥95%)	100	0.2	0.5

Chi-square = 14,320, p = NS.
CAD = coronary artery disease; LAD = left anterior descending coronary artery.

and the rates of treatment with different medications were similar across $G^{894}T$ eNOS (not shown) and $T^{-786}C$ genotypes (Tables 2 and 3). At recruitment, only 32% of the patients were on lipid-lowering treatment, and of them half were within the LDL-C goal according to the National Cholesterol Education Program criteria (21).

The $G^{894}T$ genotype distribution was GG = 20.6%, GT = 38.8%, and TT = 40.6%; the rare (G) allele frequency is shown in Table 4. The $T^{-786}C$ genotype distribution was CC = 19.2%, CT = 41.3%, and TT = 39.5%; the rare (C) allele frequency was 0.397 (Table 4). The allele frequency of both SNPs are similar to those previously reported in Caucasians (31). The linkage disequilibrium was deduced from the estimated haplotype frequencies, and its extent was expressed in term of D′ that is the ratio of the unstandardized coefficient to its maximal/minimal value (Table 4) (32). The distribution of both genotypes agreed with the Hardy-Weinberg equilibrium. No differences of genotype frequencies between the non-CAD and CAD groups were observed.

Haplotype analysis. The two genotypes for the eNOS gene resulted in four possible haplotypes, the frequencies of which are given in Table 5. The haplotype distribution did not differ between non-CAD and CAD groups. However, a significant association between haplotype T^{894}/C^{-786} and the CAD index score was found (p = 0.031) after adjustment for effects of age, creatinine, LVEF, and arterial hypertension. A

Table 2. Demographic and Clinical Characteristics of the Patients Stratified by $T^{-786}C$ eNOS Genotype

Variable	TT (n = 436, 39.5%)	TC and CC (n = 668, 60.5%)	p
Age (yrs)	63.9 ± 10.1	63.6 ± 9.5	NS
Gender (male/female)	330 (76%)/106 (24%)	487 (73%)/181 (27%)	NS
Nonsmoker/smoker/ex-smoker (%)	42/15/43	43/14/43	NS
BMI (kg/m^2)	26.9 ± 4.1	26.9 ± 3.9	NS
Serum creatinine (μmol/l)	98.6 ± 67.9	93.7 ± 54.0	NS
Serum K^+ (mmol/l)	4.3 ± 0.4	4.2 ± 0.4	NS
Serum Na^+ (mmol/l)	140 ± 3	140 ± 3	NS
Heart rate (beats/min)	68 ± 10	67 ± 10	NS
Systolic BP (mm Hg)	134 ± 17	135 ± 18	NS
Diastolic BP (mm Hg)	78 ± 10	79 ± 10	NS
Glycemia (mmol/l)	6.2 ± 2.0	6.2 ± 1.9	NS
Total cholesterol (mg/dl)	206 ± 43	205 ± 43	NS
HDL cholesterol (mg/dl)	46 ± 12	46 ± 13	NS
LDL cholesterol (mg/dl)	132 ± 36	130 ± 35	NS
Triglycerides (mg/dl)	138 ± 74	143 ± 100	NS
Left ventricular EF (%)	60 ± 15	60 ± 15	NS

Results are expressed as mean ± SD. No significant differences across genotypes were found.
BMI = body mass index; BP = blood pressure; EF = ejection fraction; eNOS = endothelial nitric oxide synthase; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

JACC Vol. 48, No. 6, 2006
September 19, 2006:1166–74

Table 3. History and Medical Treatment in the Patients Stratified by $T^{-786}C$ eNOS Genotype

History and Treatment (%)	TT (n = 436)	TC and CC (n = 668)	p
Previous CABG	9	8	NS
Previous PTCA	5	7	NS
Previous MI	32	33	NS
Antiplatelet agents	69	69	NS
Calcium channel blockers	35	39	NS
Beta-blockers	36	38	NS
Angiotensin-converting enzyme inhibitors	49	45	NS
Nitrates	59	63	NS
Warfarin	6	6	NS
Alpha-1 receptor blockers	4	4	NS
Angiotensin type I receptor blockers	6	4	NS
Diuretics	34	34	NS
Heparin	19	21	NS
Digoxin	13	13	NS
Lipid-lowering treatment	29	34	NS

Results are expressed as percentage. No significant differences across genotypes were found.

CABG = coronary artery bypass graft; MI = myocardial infarction; PTCA = percutaneous coronary angioplasty.

borderline significant association of the T^{894}/T^{-786}haplotype with CV death was also found (p = 0.066).

Biochemical markers. For these assays based on sample size calculations (nQuery Advisor version 6.0 L, Statistical Solutions, Cork, Ireland), we randomly selected 88 CC and 88 TT homozygous patients. Of these, 113 were on nitrate treatment and 63 were not. The plasma nitrate/nitrite values were similar to those previously described (33,34); however, there were no differences observed between CC and TT individuals and between patients receiving and not receiving nitrates. At variance, highly significant differences of plasma nitrotyrosine and myeloperoxidase levels between CC and TT patients were observed (Fig. 1): nitrotyrosine, which showed values similar to those seen in diabetic patients (35), was higher in CC individuals. By contrast, myeloperoxidase was lower in CC than in TT patients. All of these differences were more marked in the patients without concomitant nitrate treatment.

Follow-up data. After a median follow-up of 1,296 days (range 4 to 2,057 days), 85 CV deaths occurred. There were no significant differences in CV death rates across $G^{894}T$ genotypes by chi-square, Kaplan-Meier, and Cox regression analyses (not shown). By contrast, there were more CV deaths in the 430 TT homozygous for the $T^{-786}C$ eNOS SNP than in the 656 CC + CT individuals (43 [10%] vs. 42 [7%], respectively, chi square = 4.47, p = 0.03). Kaplan-Meier analysis confirmed the impact of the $T^{-786}C$ eNOS genotype on CV death-free survival by evidencing a significantly (p = 0.01) worse CV death-free survival in the TT than in the CT and CC individuals (Fig. 2). At Cox regression analysis, when the potential confounders of the genotype effect on outcome were considered, the significant impact of the TT eNOS genotype on CV death-free survival was consistently confirmed by using different regression models within the constraints imposed by the number of CV deaths observed. Table 6 showed the variables that remained in one such model. Of interest, the multivariate Cox regression analysis showed that the prog-

Table 4. Allele Frequencies and Pairwise Linkage Disequilibrium D′ Between $C^{894}T$ and $T^{-786}C$ Polymorphisms

Genotype	Rare Allele Frequencies	$G^{894}T$
$G^{894}T$	0.394	—
$T^{-786}C$	0.397	0.30*

*p value < 0.0001. Values were similar in the non-CAD and CAD. Pairwise linkage disequilibrium was deduced from the estimated haplotype frequencies, and its extent was expressed in term of D′, that is, the ratio of the unstandardized coefficient to its maximal/minimal value.

CAD = coronary artery disease.

Table 5. Estimated Haplotype Frequencies Under Linkage Disequilibrium in Non-CAD and CAD Patients

Haplotype	Non-CAD (CAD Index Score = 0)	CAD (CAD Index Score >0)
G^{894}/C^{-786}	0.196	0.163
G^{894}/T^{-786}	0.408	0.444
T^{894}/C^{-786}	0.242	0.221
T^{894}/T^{-786}	0.153	0.173

No significant differences of haplotype frequencies between non-CAD and CAD patients were found. Statistical values were adjusted by gender.

CAD = coronary artery disease.

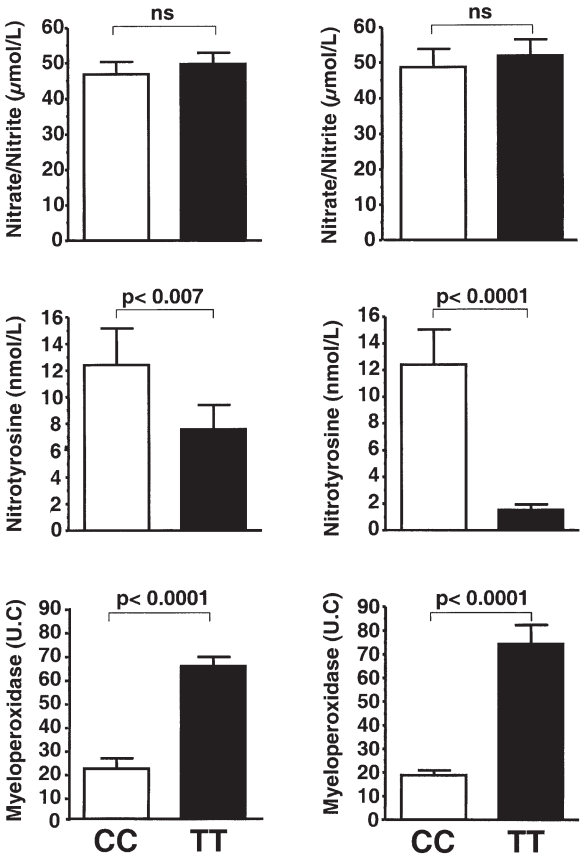

Figure 1. The histograms showed the plasma levels of nitrate/nitrite **(upper panel)**, nitrotyrosine **(middle panel)**, and myeloperoxidase **(bottom panel)** in randomly selected CC (n = 88) and TT (n = 88) patients. The **panels on the left** showed all of the cohort; those on the **right** pertain to the CC (n = 26) and TT (n = 37) patients who were not receiving nitrate for treatment of coronary artery disease (CAD).

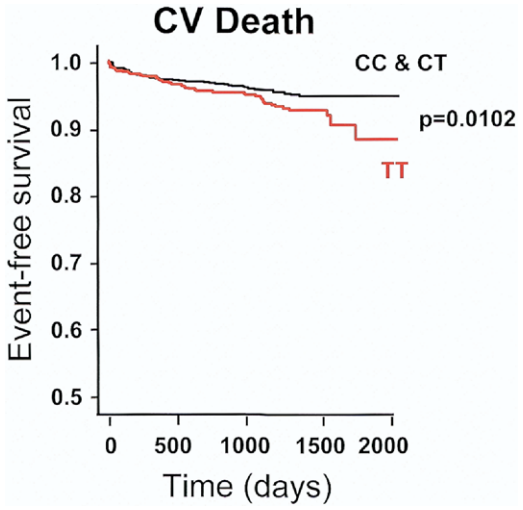

Figure 2. Results of Kaplan-Meier analysis showing cardiovascular (CV) death-free survival in the high-risk patients divided by the $T^{-786}C$ endothelial nitric oxide synthase (eNOS) genotype. The TT homozygous patients had a significantly lower CV death-free survival.

nostic impact of the TT homozygosity exceeded that of some well-established CV risk factors that did not remain in the model. To corroborate the results obtained with the Cox stepwise regression, we used the sequential regression technique described by Tabachnick and Fidell (28), which allows the researcher to control the advancement of the regression process, rather than have the statistics computed from data control entry of variables. Results obtained with both regression techniques were practically identical. They were also remarkably similar when the Cox analysis was repeated after exclusion of the patients who were on lipid-lowering treatment, thus ruling out the possibility that the small and not significant differences in the treatment rate across genotypes at baseline might have affected the eNOS genotype impact on CV death-free survival. Moreover, by performing the Kaplan-Meier and Cox analyses separately in the normotensive and hypertensive cohort, the impact of the $T^{-786}C$ eNOS genotype on CV death-free survival was found to be particularly evident in the patients with arterial hypertension.

The adverse significant impact of the TT eNOS genotype was confirmed at Kaplan-Meier analysis also on the secondary CV end point, e.g., a composite of the aforementioned CV events (not shown). At variance with these results, no significant association of the most common haplotypes (shown in Table 5) with the primary or secondary end points was found.

DISCUSSION

The identification of the genetic determinants of the susceptibility to common polygenic disorders, such as CAD and CV events, requires a careful investigative strategy, a rigorous methodological approach, a selection of biologically plausible candidate genes and, within them, of functional SNPs. Equally important, solid data can only derive from studies adequately powered from the statistical standpoint, such as the prospective survey of high-risk cohorts of patients, in whom a high rate of CV events occurs in a limited period of time.

A preliminary survey of the cross-sectional data of the cohort enrolled in the GENICA study indicated that 97% of the patients were at high CV risk according to the ATP III (National Cholesterol Education Program [Adult Treatment Panel III]) criteria (21), and had a constellation of risk factors and associated conditions that imply oxidant stress and blunted NO bioactivity. Hence, despite an LVEF that on average was normal, a very high rate of CV deaths was observed at follow-up in these CAD patients. Thus, this study was adequately powered to address the hypothesis that the eNOS variant impacted survival. Formal calculation of power by nQuery (version 6.0) confirmed this prediction by showing that given our sample size, a total number of 79 events, e.g., smaller than that actually observed, provided a 95% power to detect a 6% difference of survival curves between a CC + CT and TT genotype groups at a 0.05-level two-sided log-rank test, assuming no drop-outs.

$G^{894}T$ exon 7 eNOS SNP and survival. We found no evidence for an effect of the $G^{894}T$ SNP on total and CV death with different analyses. This negative finding contrasts with results of two studies that reported a correlation with MI (36,37), but accords well with the conclusions of a prospective study of high-risk Japanese nondiabetic hemodialysis patients (38), and also with previous evidence for no association of this SNP with the severity of CAD (14,16,31,39–41). It also accords with the fact that the Glu298Asp is a conservative replacement that may have no functional effect (12). Moreover, studies in vivo in humans suggested that it might affect NO bioactivity only through an interaction with the $T^{-786}C$ SNP (13), likely because the 2 SNPs are in linkage disequilibrium as shown in Table 4.

$T^{-786}C$ promoter eNOS SNP and survival. Our results showed that the $T^{-786}C$ SNP in the eNOS gene promoter had a significant impact on CV death-free survival: the survival curve of the TT patients started to diverge after a lag-phase of about 500 days and continued to do so afterward (Fig. 2). Of interest, the haplotype analysis also suggested a worse CV death-free survival in patients with the T^{-786} allele, e.g., with the T^{894}/T^{-786}haplotype. Reporter gene studies showed that this $T^{-786}C$ substitution

Table 6. Cardiovascular Death-Free Survival at Cox Regression Analysis

Variables	p	Odds Ratio	95% Confidence Interval
Left ventricular ejection fraction	<0.0001	0.95	0.93–0.96
Age	0.002	1.05	1.02–1.09
$T^{-786}C$ genotype (CC + CT vs. TT)	0.006	2.14	1.24–3.67

The variables that remained in the model are reported along with significance values and 95% confidence interval. The following variables did not remain in the model: the $G^{894}T$ eNOS SNP, gender, arterial hypertension, LDL cholesterol, smoking status, serum creatinine, diabetes mellitus, coronary artery atherosclerotic burden and (CAD Duke Index).

Abbreviations as in Table 2.

markedly blunts the transcription rate of the eNOS gene (42), and hence NO production, likely because the C allele creates a binding site for a replication protein A1 that acts as a suppressor of eNOS transcription (43). We previously observed a blunted NO bioactivity in vivo in hypertensive patients with the C allele (13), and these results were thereafter confirmed by the demonstration that the C allele implies a blunted eNOS transcription in response to shear stress (15), which is a crucial determinant of basal NO production (44). Notwithstanding these functional data, we could find no evidence for an effect of this SNP on nitrate/nitrite plasma levels (Fig. 1) in keeping with previous albeit not all findings (31,33). The fact that this measurement is affected by dietary factors and by contributions of other NOS enzymes can well account for these results (31). Thus, although available findings overall are consistent with a relevant biological role of the $T^{-786}C$, our findings show for the first time an effect of this SNP on survival in high-risk individuals.

Potential explanations for the impact of the T^{-786} allele on survival. The association of gene variants with complex phenotypes such as CAD and CV events, which involve numerous mechanisms and therefore have multiple determinants, can be affected by several confounders. These deserve careful consideration when analyzing of the results because overlooking them can lead to misleading conclusions. As an example, the coronary atherosclerotic burden differs among patients carrying the different $T^{-786}C$ genotypes (14–16). Furthermore, in this type of study, the distribution of major risk factors, associated CV conditions, and pharmacological treatment is usually unbalanced between the patients experiencing and those not experiencing CV events. Therefore, we used a hierarchical sequential type of Cox regression analysis (28) in which the major determinants of outcome, e.g., LVEF, coronary atherosclerotic burden (24,25), gender, age, arterial hypertension, hypercholesterolemia, smoking status, serum creatinine, diabetes mellitus, and rate of lipid lowering treatment, were considered. This technique, which allows control over the advancement of the regression process (28), fully confirmed the results obtained with a classic stepwise regression analysis. Thus, results obtained with either technique support an independent significant impact of the eNOS promoter SNP on CV death. This is important novel information that, in our view, is even more striking given the high-risk features of this cohort. Moreover, the impact of the $T^{-786}C$ genotype on CV death remained significant when the modified Duke Prognostic Index was handled either as categorical or as a continuous variable and also when the rate of lipid-lowering treatment was considered in the Cox analysis.

The worse CV death-free survival of the TT homozygous as compared to the other eNOS genotypes (Fig. 2) is intriguing given the aforementioned association of the C allele with blunted NO bioactivity (13), and with multivessel CAD (14,16). Notably, a significant association of the C allele with CAD was also confirmed by our present analysis, in which the T^{894}/C^{-786} haplotype correlated significantly with the CAD index score. Moreover, an association of the ^{-786}C allele with CV events in Japanese hemodialysis patients has also been recently described (37). The race differences between the latter patients and our cohort are obvious; furthermore, patients with end-stage renal disease are likely to have an accumulation of the endogenous NO inhibitor asymmetric dimethylarginine (45). Hence, a deleterious impact of the genetic predisposition to generate more NO is unlikely in these patients. Moreover, our present finding that the T^{-786} allele, which can imply a higher NO bioactivity, is associated with increased CV death, along with the recently reported association of the same allele with previous MI in Caucasians (46), seems to be paradoxical. Nonetheless, along with the conflicting results in this area of research (47), it raises some provocative questions on the potential clinical importance of the genetic predisposition to generate NO in the long run.

It must also be acknowledged that the determinants of plaque growth, e.g., of the atherosclerotic burden, and of plaque destabilization, e.g., of CV events, differ even though they can overlap. Fatal MI and atherothrombotic stroke are commonly triggered by rupture of vulnerable atherosclerotic plaques, with the propensity of rupture enhanced by several factors involving matrix metalloproteinase (MMP) activation. Of note, peroxynitrite ($ONOO^-$), originating from NO and superoxide anion (48), can activate MMPs in plaques, thus contributing to plaque destabilization (49,50). Furthermore, it was recently shown that oxidized LDL cause eNOS uncoupling and increase superoxide anion production in endothelial cells in vitro by inhibiting protein kinase C-alpha and thereby blunting the phosphorylation of eNOS Thr^{495} (51).

The GENICA study enrolled high-CV-risk patients in whom enhanced oxidant stress is the rule rather than the exception: they were chronically exposed to oxidatively modified lipoproteins (20), and therefore might have uncoupling of eNOS. Accordingly, the predisposition to generate more NO of the homozygous TT can result into a higher generation of ROS and reactive nitrogen species (RNS), including $ONOO^-$, with ensuing MMP activation. In addition, it has been shown that chronic exposure to $ONOO^-$ irreversibly oxidizes relevant cysteine thiols in sarcoplasmic/endoplasmic reticulum Ca^{2+} adenosine triphosphatase, thus blocking Ca^{2+} uptake and thereby impairing NO-dependent arterial relaxation (52). Accordingly, under conditions with enhanced ROS/RNS generation, a genetic predisposition to generate more NO might be detrimental in the long run. In fact, it might result in impairment of both sarcoplasmic/endoplasmic reticulum Ca^{2+} adenosine triphosphatase activity and arterial dilatation, and activation of MMPs, with ensuing plaque rupture, atherothrombosis, and fatal CV events.

To gain some mechanistic insight, we measured two markers of oxidant stress in plasma, e.g., nitrotyrosine and myeloperoxidase. The former is held to derive from $ONOO^-$ effect on protein tyrosine residues, and the latter reflects oxidant burst in polymorphonuclear neutrophils and was found to be associated with CAD (53,54). Our results showed that nitrotyrosine levels were markedly higher in the CC compared with the TT individuals (Fig. 1), in contrast with our expectations. Although indicating that the plasma levels of this marker do not accurately reflect NO bioactivity of CC individuals (13), these results may suggest NO consumption in the TT patients. Notably, the fact that these patients concomitantly had markedly higher myeloperoxidase values than CC patients (Fig. 1), along with the fact that myeloperoxidase consumes NO (54), might account for the lower nitrotyrosine levels. It can explain, at least in part, the worse CV death-free survival in this genotype, because myeloperoxidase activates MMPs and was found to predict risk of CV events in patients with acute coronary syndromes (54). Thus, our results overall suggest a link between the $T^{-786}C$ eNOS genotype and leucocyte activation, leading to increased ROS/RNS generation locally with atherosclerotic plaque. Further investigation is obviously necessary to clarify its nature.

Finally, although the possibility of a serendipitous finding can never be totally excluded in clinical research, we would like to underline that prospective cohort studies, such as the present one, have a stronger design and therefore usually provide more robust conclusions than cross-sectional studies, which are exposed to several potential biases that might affect results and conclusions (55).

Conclusions. Our results shown that in a high-risk population of consecutive patients referred for coronary angiography, a functional polymorphism of the promoter region of the eNOS gene predicted CV death independent of other common CV risk factors and of the CAD atherosclerotic burden. They also documented clear-cut differences of plasma nitrotyrosine and myeloperoxidase levels between CC and TT homozygous patients. These findings collectively raise provocative questions regarding the importance of the genetic predisposition to generate NO in these patients that should prompt further research on the molecular mechanisms that are involved in atherosclerotic plaque erosion and rupture and imply NO bioactivity and oxidant stress.

Acknowledgment

The authors are grateful to Dr. David Tregouet for his help with the Thesias software.

Reprint requests and correspondence: Dr. Gian Paolo Rossi, Clinica Medica 4, University Hospital, via Giustiniani, 2, 35126 Padova, Italy. E-mail: gianpaolo.rossi@unipd.it.

REFERENCES

1. American Heart Association. Heart Disease and Stroke Statistics—2004 Update. Available at: http://www.americanheart.org/downloadable/heart/1079736729696HDSStats2004UpdateREV3-19-04.pdf. Accessed January 9, 2006.
2. Wang Q, Rao S, Shen GQ, et al. Premature myocardial infarction novel susceptibility locus on chromosome 1P34-36 identified by genomewide linkage analysis. Am J Hum Genet 2004;74:262–71.
3. Wang L, Fan C, Topol SE, Topol EJ, Wang Q. Mutation of MEF2A in an inherited disorder with features of coronary artery disease. Science 2003;302:1578–81.
4. Helgadottir A, Manolescu A, Thorleifsson G, et al. The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. Nat Genet 2004;36:233–9.
5. Winkelmann BR, Hager J, Kraus WE, et al. Genetics of coronary heart disease: current knowledge and research principles. Am Heart J 2000;140:S11–S26.
6. Al Suwaidi J, Hamasaki S, Higano ST, et al. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation 2000;101:948–54.
7. Targonski PV, Bonetti PO, Pumper GM, et al. Coronary endothelial dysfunction is associated with an increased risk of cerebrovascular events. Circulation 2003;107:2805–9.
8. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 2000;101:1899–906.
9. Halcox JP, Schenke WH, Zalos G, et al. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002;106:653–8.
10. Hingorani AD. Polymorphisms in endothelial nitric oxide synthase and atherogenesis: John French Lecture 2000. Atherosclerosis 2001; 154:521–7.
11. Tesauro M, Thompson WC, Rogliani P, et al. Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: cleavage of proteins with aspartate vs. glutamate at position 298. Proc Natl Acad Sci USA 2000;97:2832–5.
12. Fairchild TA, Fulton D, Fontana JT, et al. Acidic hydrolysis as a mechanism for the cleavage of the Glu(298)→Asp variant of human endothelial nitric-oxide synthase. J Biol Chem 2001;276:26674–9.
13. Rossi GP, Taddei S, Virdis A, et al. The T-786C and Glu298Asp polymorphisms of the endothelial nitric oxide gene affect the forearm blood flow responses of Caucasian hypertensive patients. J Am Coll Cardiol 2003;41:938–45.
14. Rossi GP, Cesari M, Zanchetta M, et al. The T-786C endothelial nitric oxide synthase genotype is a novel risk factor for coronary artery disease in Caucasian patients of the GENICA study. J Am Coll Cardiol 2003;41:930–7.
15. Cattaruzza M, Guzik TJ, Slodowski W, et al. Shear stress insensitivity of endothelial nitric oxide synthase expression as a genetic risk factor for coronary heart disease. Circ Res 2004;95:841–7.
16. Hirashiki A, Yamada Y, Murase Y, et al. Association of gene polymorphisms with coronary artery disease in low- or high-risk subjects defined by conventional risk factors. J Am Coll Cardiol 2003;42:1429–37.
17. Celermajer DS, Sorensen KE, Spiegelhalter DJ, et al. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. J Am Coll Cardiol 1994;24:471–6.
18. Luscher TF, Tanner FC, Tschudi MR, Noll G. Endothelial dysfunction in coronary artery disease. Ann Rev Med 1993;44:395–418.
19. Casas JP, Bautista LE, Humphries SE, Hingorani AD. Endothelial nitric oxide synthase genotype and ischemic heart disease: meta-analysis of 26 studies involving 23028 subjects. Circulation 2004;109: 1359–65.
20. Rossi GP, Cesari M, De Toni R, et al. Antibodies to oxidized low-density lipoproteins and angiographically assessed coronary artery disease in white patients. Circulation 2003;108:2467–72.
21. Cesari M, Maiolino G, Colonna S, et al. Under treatment with lipid-lowering drugs of high-risk coronary heart disease patients of the GENICA study. J Cardiovasc Pharmacol 2003;42:484–90.
22. 2003 European Society of Hypertension–European Society of Cardiology guidelines for the management of arterial hypertension. J Hypertens 2003;21:1011–53.

23. Cesari M, Zanchetta M, Burlina A, et al. Hyperhomocysteinemia is inversely related with left ventricular ejection fraction and predicts cardiovascular mortality in high-risk coronary artery disease hypertensives. Arterioscler Thromb Vasc Biol 2005;25:115–21.
24. Smith LR, Harrell FE Jr., Rankin JS, et al. Determinants of early versus late cardiac death in patients undergoing coronary artery bypass graft surgery. Circulation 1991;84:III245–53.
25. Mark DB, Nelson CL, Califf RM, et al. Continuing evolution of therapy for coronary artery disease. Initial results from the era of coronary angioplasty. Circulation 1994;89:2015–25.
26. Califf RM, Armstrong PW, Carver JR, D'Agostino RB, Strauss WE. 27th Bethesda Conference: matching the intensity of risk factor management with the hazard for coronary disease events. Task Force 5. Stratification of patients into high, medium and low risk subgroups for purposes of risk factor management. J Am Coll Cardiol 1996;27:1007–19.
27. Staessen JA, Fagard R, Thijs L, et al. Randomised double-blind comparison of placebo and active treatment for older patients with isolated systolic hypertension. The Systolic Hypertension in Europe (Syst-Eur) Trial Investigators. Lancet 1997;350:757–64.
28. Tabachnick BG, Fidell LS. Multiple regression. In: Tabachnick BG, Fidell LS, editors. Using Multivariate Statistics. Boston, MA: Allyn and Bacon, 2001:133–9.
29. GeneCanvas. Thesias. Available at: http://ecgene.net/genecanvas/news.php. Accessed March 6, 2006.
30. Tregouet DA, Escolano S, Tiret L, Mallet A, Golmard JL. A new algorithm for haplotype-based association analysis: the Stochastic-EM algorithm. Ann Hum Genet 2004;68:165–77.
31. Jeerooburkhan N, Jones LC, Bujac S, et al. Genetic and environmental determinants of plasma nitrogen oxides and risk of ischemic heart disease. Hypertension 2001;38:1054–61.
32. Tregouet DA, Tiret L. Cox proportional hazards survival regression in haplotype-based association analysis using the Stochastic-EM algorithm. Eur J Hum Genet 2004;12:971–4.
33. Wang XL, Mahaney MC, Sim AS, et al. Genetic contribution of the endothelial constitutive nitric oxide synthase gene to plasma nitric oxide levels. Arterioscler Thromb Vasc Biol 1997;17:3147–53.
34. Koh KK, Ahn JY, Choi YM, et al. Vascular effects of step I diet in hypercholesterolemic patients with coronary artery disease. Am J Cardiol 2003;92:708–10.
35. Wang XL, Rainwater DL, Leone A, Mahaney MC. Effects of diabetes on plasma nitrotyrosine levels. Diabet Med 2004;21:577–80.
36. Shimasaki Y, Yasue H, Yoshimura M, et al. Association of the missense Glu298Asp variant of the endothelial nitric oxide synthase gene with myocardial infarction. J Am Coll Cardiol 1998;31:1506–10.
37. Hingorani AD, Liang CF, Fatibene J, et al. A common variant of the endothelial nitric oxide synthase (Glu298→Asp) is a major risk factor for coronary artery disease in the UK. Circulation 1999;100:1515–20.
38. Asakimori Y, Yorioka N, Tanaka J, et al. Association between ENOS gene polymorphism and cardiovascular events in nondiabetic hemodialysis patients: a prospective study. Am J Kidney Dis 2004;44:112–20.
39. Hibi K, Ishigami T, Tamura K, et al. Endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction. Hypertension 1998;32:521–6.
40. Poirier O, Mao C, Mallet C, et al. Polymorphisms of the endothelial nitric oxide synthase gene—no consistent association with myocardial infarction in the ECTIM study. Eur J Clin Invest 1999;29:284–90.
41. Gardemann A, Lohre J, Cayci S, et al. The T allele of the missense Glu(298)Asp endothelial nitric oxide synthase gene polymorphism is associated with coronary heart disease in younger individuals with high atherosclerotic risk profile. Atherosclerosis 2002;160:167–75.
42. Nakayama M, Yasue H, Yoshimura M, et al. T-786→C mutation in the 5′-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. Circulation 1999;99:2864–70.
43. Miyamoto Y, Saito Y, Nakayama M, et al. Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a −786T→C mutation associated with coronary spastic angina. Hum Mol Genet 2000;9:2629–37.
44. Luscher TF, Corti R. Flow: the signal of life. Circ Res 2004;95:749–51.
45. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 1992;339:572–5.
46. Agema WR, DeMaat MP, Zwinderman AH, et al. An integrated evaluation of endothelial constitutive nitric oxide synthase polymorphisms and coronary artery disease in men. Clin Sci (Lond) 2004;107:255–61.
47. Hingorani A. Resolving inconsistency in the results of genetic association studies of cardiovascular disease. Clin Sci (Lond) 2004;107:251–3.
48. Okamoto T, Akaike T, Sawa T, et al. Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. J Biol Chem 2001;276:29596–602.
49. Siwik DA, Pagano PJ, Colucci WS. Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts. Am J Physiol Cell Physiol 2001;280:C53–C60.
50. Siwik DA, Colucci WS. Regulation of matrix metalloproteinases by cytokines and reactive oxygen/nitrogen species in the myocardium. Heart Fail Rev 2004;9:43–51.
51. Fleming I, Mohamed A, Galle J, et al. Oxidized low-density lipoprotein increases superoxide production by endothelial nitric oxide synthase by inhibiting PKCalpha. Cardiovasc Res 2005;65:897–906.
52. Adachi T, Weisbrod RM, Pimentel DR, et al. S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. Nat Med 2004;10:1200–7.
53. Zhang R, Brennan ML, Fu X, et al. Association between myeloperoxidase levels and risk of coronary artery disease. JAMA 2001;286:2136–42.
54. Baldus S, Heeschen C, Meinertz T, et al. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. Circulation 2003;108:1440–5.
55. Rossi GP, Maiolino G. Do meta-analyses of association studies of endothelial nitric oxide synthase variants and ischemic heart disease provide conclusive answers? Circulation 2004;110:e305–6.